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ORIGINAL ARTICLE

2.45-GHz microwave irradiation adversely affects reproductive function in male mouse, *Mus musculus* by inducing oxidative and nitrosative stress

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Abstract

Electromagnetic radiations are reported to produce long-term and short-term biological effects, which are of great concern to human health due to increasing use of devices emitting EMR especially microwave (MW) radiation in our daily life. In view of the unavoidable use of MW emitting devices (microwaves oven, mobile phones, Wi-Fi, etc.) and their harmful effects on biological system, it was thought worthwhile to investigate the long-term effects of low-level MW irradiation on the reproductive function of male Swiss strain mice and its mechanism of action. Twelve-week-old mice were exposed to non-thermal low-level 2.45-GHz MW radiation (CW for 2 h/day for 30 days, power density = 0.029812 mW/cm^2 and SAR = 0.018 W/Kg). Sperm count and sperm viability test were done as well as vital organs were processed to study different stress parameters. Plasma was used for testosterone and testis for 3β HSD assay. Immunohistochemistry of 3β HSD and nitric oxide synthase (i-NOS) was also performed in testis. We observed that MW irradiation induced a significant decrease in sperm count and sperm viability along with the decrease in seminiferous tubule diameter and degeneration of seminiferous tubules. Reduction in testicular 3β HSD activity and plasma testosterone levels was also noted in the exposed group of mice. Increased expression of testicular i-NOS was observed in the MW-irradiated group of mice. Further, these adverse reproductive effects suggest that chronic exposure to nonionizing MW radiation may lead to infertility via free radical species-mediated pathway.

Keywords: microwave irradiation, oxidative stress, free radicals, antioxidant enzymes, steroidogenesis, reproduction

Introduction

In recent years, there has been many-fold increase in the strength of electromagnetic radiation (EMR) in our environment. EMR is caused by the escape of electromagnetic waves (EMWs) which are produced by the oscillations of electrically charged particles in free space. According to its frequency and energy, EMR can be classified into ionizing radiation (IR) or nonionizing radiation (NIR). IRs involve EMWs of extremely high frequency such as X-rays and gamma rays. These EMWs have enough photon energy to produce ionization by breaking the atomic bonds that hold molecules together in cells. NIR involves the EMWs especially microwaves (MWs) having wavelength from 1 mm to 1 m and frequency between 0.3 and 300 GHz. The photon energy of the NIR is too weak to break the atomic bonds or to produce ionization of molecules. MW radiation can produce thermal effect by inducing electric currents and alter chemical reactions in the cells and tissues. In addition to this, nonthermal effects of MW radiation have also been identified. MW radiations are reported to produce various long-term and short-term biological effects, which are of great concern to human health due to the increasing use of MW-emitting devices in our daily life such as MW oven (2.45 GHz), mobile phones (900/1800/2100 MHz), etc. It has been reported that the leakage from MW oven is 5 mW/cm² (power density) at 5 cm (estimated SAR value 0.256 W/kg) and 1.5 mW/cm² at 30 cm (estimated SAR value of 0.0056 W/kg) [1]. This suggests that the power density of the MW radiation decreases rapidly with increasing distance from the MW-emitting devices.

International Agency for Research on Cancer (IARC) classified EMRs as possible carcinogen to humans. People heavily exposed to MW radiations are more prone to nonmalignant tumors and had an increased risk of malignant gliomas and higher rate of acoustic neuromas. Due to health concern issues, some relevant National and International organizations came forward to establish guidelines, legislations and to recommend exposure limits for safe uses. International Council for Non-Ionizing Radiation Protection and the Institute of Electrical and Electronics Engineers (IEEE) published recommendations concerning the levels of exposure for the general public and workers [2,3]. These guidelines result from the various studies on internal electric fields, currents and MW energy depositions within biological tissues during exposures.

Recent studies elucidated that MW radiation may exert a wide range of adverse effects on the biological system. Exposure to these radiations affect the activation of peritoneal macrophages to a virucidal state, increased immune

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response [4–6], modification of the lipid peroxidation conditions [7–9] etc. The low-level nonthermal MW radiation or mobile phone radiation may propagate through the body and affect the reproductive system of both males and females. MW radiation emitted from mobile phones are also reported to exert an adverse effect on the pituitary gonadotropins, gonadal steroids, sperm count, motility, viability, and quality as well as ROS level and total antioxidant capacity (TAC) capacity of semen [10–14]. Our laboratory has reported that in female mice, 2.45-GHz low-level continuous wave (CW) MW radiation affects embryo implantation sites and causes DNA damage in the brain cell of mice [15]. We have also found that nonthermal MW irradiation-induced oxidative stress not only suppresses implantation, but may also lead to implantation failure/resorption and deformity of the embryo in case pregnancy continues [16]. Further, it has been reported that MW exposure may exert a wide range of adverse effects on the testicular function. Radiofrequency (RF) field exposure to anaesthetized rats and mice resulting in elevated testicular or body temperature may cause depletion of the spermatogenic epithelium, abnormality in sperm morphology, reduction in sperm count, and primary spermatocyte resulting in decreased fertility [17–20]. Studies also suggest that exposure to the various RF fields at different level (1.7 and 10 mW m^{-2}) from a commercial antenna park produced a rapid drop in fertility in mice [21]. Akdag et al. [22] reported decreased epididymal sperm count and increased percentage of abnormal sperm in rats chronically exposed to 9.45 GHz at a whole-body SAR of about 2 W/kg.

MW radiation acts as one of the environmental stressors, responsible for generating/inducing oxidative stress, a pronounced prooxidant state, caused by the excessive production of free radicals or from the weakening of the antioxidant defense system. Imbalance between the production and manifestation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), that is, extensive production of free radicals, cause toxic effects and damage all the components of a cell, including proteins, lipids, and nucleic acids. Free radicals are also implicated in the induction of apoptosis [23] and cellular senescence [24]. It promotes the oxidation of amino acid residue side chains, formation of protein–protein cross-linkages and oxidation of the protein backbone resulting in fragmentation and aggregation. These oxidative modifications enhance the degradation of critical enzymes by the multicatalytic proteasome complex [25] and thereby results in havoc rise in free radicals throughout the cell lead to oxidative stress.

Both oxidative and nitrosative stress have been attributed to most of the cases of infertility of both males and females [26]. Male infertility, which currently account for approximately half of all cases, is increasing faster than female infertility. Despite the low oxygen tensions that characterize the testicular microenvironment, testis remains vulnerable to oxidative and nitrosative stress due to the abundance of highly unsaturated fatty acid and the presence of potential ROS/RNS generating system. Substantial evidence suggests that small amount of free radicals are necessary for spermatozoa to acquire fertilizing capabilities [27,28]. Low levels of free radicals are essential for acrosome reaction, hyperactivation, motility, capacitation, and fertilization [29,30]. But at high concentration it damages the sperm membrane which in turn reduces the sperm's motility and ability to fuse with the oocyte. ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. MW radiation emitted from mobile phones may produce ROS which is responsible for inducing infertility [31]. Studies suggest that sperm damage by ROS may occur due to MW field exposure [32–34]. MW exposure at 2.45 GHz decreases sperm count, increases apoptosis, and affects the level of antioxidant enzymes [35]. The increased ROS/RNS production in the testis is responsible for possible toxic effects on physiology of reproduction. However, cells have their defense mechanism, that is, antioxidants (glutathione peroxidase-GPx, catalase-CAT, and superoxide dismutase-SOD) to fight against increased production of ROS. Further, Kesari et al. [36–39] have shown changes in the antioxidant enzyme levels at various MW frequencies (900 MHz, 2.45 GHz, and 50 GHz) in brain and sperm.

Although majority of studies on MW-irradiated animals with reference to reproductive performance are associated with sperm deformities but the underlying mechanism is still unknown. In view of the unavoidable use of MW-emitting devices and their harmful effects on the biological system, it was thought worthwhile to investigate the long-term effects of low-level MW exposure (2.45 GHz) on antioxidant system and ROS/RNS generation as well as alteration in the reproductive function of male mice. For our experiment, we have selected 2.45 GHz frequency which is within the range of 2.45 GHz frequency band allocated for industrial, scientific, medical, and domestic (ISMD) applications and for several wireless communication standards [40]. We have chosen the whole body specific absorption rate (SAR) level of 0.018 W/Kg, which is also comparatively very low as compared with the amount of daily exposure by MW radiation emitting appliances such as mobile phones and other appliances. Present study, for the first time indicates the ROS/NO status in different tissues as well as testicular expression of nitric oxide synthase (i-NOS) and 3β HSD of MW-exposed mice.

Materials and methods

Study design

Swiss strain male mice (12 weeks old, body weight ~30 g) were obtained from the mice colony of our laboratory maintained under light:dark cycle (LD12:12). Animals were kept under steady-state, received standard pelleted food, and water *ad libitum*. Randomly selected, 40 mice were divided into two groups (control and experimental) of 20 mice each ($N = 20$). Experimental group mice were

irradiated to 2.45-GHz CW low-level MW radiation for 2 h/day, from 09:00 h to 11:00 h for 30 days continuously. The control group mice were subjected to sham exposure. Rectal temperature of both the groups was measured before and after the exposure during the first 3 days and the last 3 days of exposure. Experiment was conducted in accordance with institutional practice and within the framework of the revised animals (Scientific procedures) act of 2002 of the Government of India on Animal welfare. Experiment was repeated twice and the native PAGE assays were repeated five times for each experiment.

All the biochemical parameters, sperm count, and sperm viability test were performed in the tissue of 15 animals while the tissues of other five animals were fixed for routine histology and immunohistochemistry. In the first experiment, brain and testis of only one mouse were fixed while in the second experiment, tissues of four mice were fixed for histology and immunohistochemical study.

Exposure setup

MW power to pyramidal horn antenna was delivered by the Analog Signal Generator MW source (Model no. E 8257D PSG, manufactured by Agilent Technologies, USA). The experimental setup used for MW irradiation of mice consists of Analog Signal Generator covering a frequency range from 250 kHz–20 GHz, a coaxial attenuator, MW amplifier (model no. 8349B Hewlett Packard Co., USA), coaxial to waveguide transition, 20 dB cross-coupler and E-plane bend, pyramidal horn antenna in addition to animal cage and a box covered by MW absorbers (Figure 1). Pyramidal horn antenna was made up of silver-polished brass material. Its throat and mouth dimensions were 7.2×3.2 cm and 9.0×5.0 cm, respectively, and axial length of the antenna from the throat was 10.0 cm. Gain of the horn antenna (G_t) was found to be 4.0657 dB, as calculated by three-antenna method. The distance between pyramidal horn and the mid-plane of animal body (assuming the body length to remain horizontal during exposure) was estimated through far-field criterion ($R \geq 2D^2/\lambda$) to be 25 cm (Figure 1). The animal cage cross-sectional dimensions in E- and H-planes (electric and magnetic field planes respectively) were designed on the basis of 1.24 and 1.69 dB beam-widths in corresponding planes of the horn, respectively. For this purpose, the horn radiation patterns were also measured at 2.45 GHz in E- and H-planes [41]. Maximum output power measured by the power meter with power sensor (Model no. 836A, manufactured by Agilent Technologies, USA) was 19.8 dBm delivered to the horn antenna from the amplifier. The power meter has a range from 30 μW to 3 W (Model no. 8481H, Hewlett Packard Co., USA).

Animal cage was designed in such a way that it contained 10 compartments of equal sizes. The dimension of the animal cage was $19.2 \times 17.6 \times 15$ cm and each compartment was $3.5 \times 8.5 \times 15$ cm accommodating single mouse at a time. Pine wood material with low dielectric constant was used for making the cage and its partitions. For air ventilation, small circular holes of 1 cm diameter were drilled on the side and the partition walls of the cage and the upper portion of the cage remained open. Base as well as sides and partition walls of the cage were covered by carbon-impregnated Styrofoam MW absorber to reduce the reflections of scattered beam. Each mouse was kept in the separate compartment of the cage throughout the exposure period. The partitions were made in such a way that mice remain restrained in the cage during the period of MW irradiation and were exposed with their length parallel to E-field. The animal cage was placed on the stand in a box covered from all the four sides by MW absorbers to minimize reflections (Figure 1). Mice were exposed daily for two hours, from 09:00 to 11:00 for 30 days continuously. The temperature in the chamber was maintained at 25–27°C throughout the exposure period by circulating air. The 20-dB cross-coupler and power meter with power sensor were used to measure the power input to antenna and reflections from it. Power transmitted from the antenna was equal to 64.776 mW and this was estimated by subtracting the power reflected from the antenna (when it faces toward free space), from the measured input power. The power density was computed using the following formula:

$$\text{Power Density} = \frac{P_t G_t}{4\pi R^2}$$

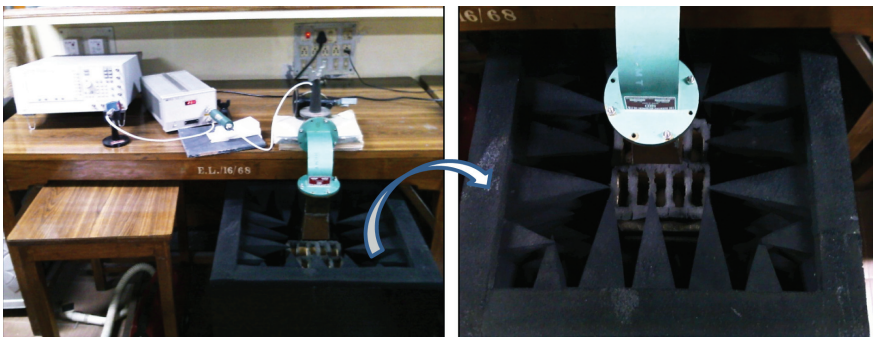

Figure 1. Microwave (MW) exposure setup and the position of animal cages during MW exposure. Mice were exposed to nonthermal 2.45-GHz low-level MW radiation for 2 h/day continuously for 30 days using MW source and Pyramidal horn antenna (power density = 0.029812 mW/cm^2 and SAR = 0.018 W/Kg).

where P_t is the power transmitted in to the cage, G_t is the gain of the horn, and R is the distance between the horn aperture and mid-plane of the body of mouse in the exposure cage. Mice of experimental group were exposed to 2.45-GHz MW radiation at power density = 0.029812 mW/cm^2 and average specific absorption rate (SAR) = 0.018 W/Kg. The SAR value was estimated for body length parallel to the electric field, as per actual placement of the mouse [41]. The exposure at power density = 0.029812 mW/cm^2 did not cause any elevation in the ambient temperature of the cage as well as rectal temperature of the mouse. The average rectal temperature of the mice was 35.81 ± 0.2°C (control group) and 35.9 ± 0.2°C (experimental group) before exposure and 35.91 ± 0.2 (control group) and 36.05 ± 0.2 (experimental group) after the end of exposure.

Blood collection and tissues sampling

After 24 h of the termination of 30 days exposure, mice were sacrificed by decapitation, and blood was collected in heparinized tubes. Brain was dissected out to separate hypothalamus and liver, kidney, and the testis were removed from the dissected body. After washing with ice-cold sterile physiological saline solution all the tissues were stored in − 20°C until processed for study of different parameters. Epididymis was removed to study sperm count and sperm viability. Brain and right testis of five animals were fixed in 4% paraformaldehyde solution for normal histology and Immunohistochemistry.

Histology of testis

Testis was fixed in 4% paraformaldehyde solution for 24 h, dehydrated through increasing concentrations of ethanol, and embedded in paraffin prior to sectioning. 6-µm-thick sections of testis were cut by Lieca RM2125 RT rotatory microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and processed for routine histology (haematoxylin-eosin staining) followed by morphometric measurements and image analysis.

Sperm viability and sperm count

Sperm viability was assessed using a supra-vital staining technique based on the principle that cells with damaged plasma membrane take up the stain, while viable ones do not [42]. For this, excised cauda epididymis placed in a pre-warmed Petri dish containing 0.5 ml of normal saline (0.9% NaCl) maintained at 37°C was minced with scalpel for approx. 1 min. A drop of sperm suspension and a drop of eosin-negrosin stain (1% eosin Y + 5% negrosin, 1:1) were placed on a clear glass slide and mixed thoroughly with the help of a fine glass rod. A portion of the mixture was transferred to a second slide and a thin film was prepared. The slide was then examined under the microscope (400×), and one hundred spermatozoa (viable and dead) were counted from 10 different areas on the slide. Spermatozoa appearing pinkish (stained) were considered to be dead, while those appearing colorless (unstained) were counted as viable. Sperm viability was calculated in percent using the following formula:

$$\text{Viability}\,(\%) = \frac{\text{number of viable spermatozoa}}{\text{total number of spermatoza (viable + dead)}} \times 100$$

Sperm count was performed using hemocytometer (Fein-Optik, Jena, Germany) with improved Neubauer ruling [42]. For this, the sperm suspension was diluted 20-fold with normal saline (0.9% NaCl) in a white blood cell pipette; the sperm suspension was drawn to the 0.5 mark halfway up the stem and saline subsequently to the 11 mark at the top of the bubble chamber. The preparation was mixed thoroughly and then one drop of it was added to both sides of the hemocytometer. The number of spermatozoa was counted in the four corner squares of the hemocytometer under a microscope (400×) and the average numbers were calculated. If spermatozoa crossed the lines of the grid, only those at the top and right-hand sides of the squares were counted. Number of spermatozoa per cauda epididymis was expressed as follows:

$$\begin{aligned}\text{Sperm number} &= \text{averaged no. of spermatozoa counted (N)} \\ &\times \text{multiplication factor (10,000)} \\ &\times \text{dilution factor (20)} = \text{N} \times 10{,}000 \\ &= \text{N} \times 0.2 \times 10^6 \text{ spermatozoa}\end{aligned}$$

Isolation of plasma and tissue homogenization

Blood samples were kept at room temperature for 30 min and then centrifuged at 4000 rpm at 4°C for 15 min. The supernatant was collected as plasma and used for testosterone estimation. For NO and ROS estimation, liver, kidney, hypothalamus, and testis were homogenized in 50 mM phosphate buffer (pH 7.4) for 30 s using a Polytron homogenizer. The remaining homogenates, after adding protease inhibitor 0.2 µM PMSF, were centrifuged at 12,000 rpm for 30 min at 4°C. The resultant supernatant was used for measuring MDA level and determining antioxidant enzymes activity by native gel. Total protein concentration was measured using Lowry's method using BSA as standard.

Measurement of total ROS level

The total ROS generation in fresh homogenate of different tissues was assessed using the method of Bejma et al. [43] with slight modification. For this, 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a nonpolar compound was used as a probe. DCFH-DA after conversion to a polar derivative by intracellular esterase, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein. Briefly, the homogenate was diluted in PBS to obtain a concentration of 25 µg tissue protein/ml. The reaction mixture containing diluted homogenate and 10 µl of DCFH-DA (10 µM) was incubated for 15 min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group

cleaved by esterase. After 30 min of further incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured using a spectrofluorimeter with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-DA in the absence of homogenate) was corrected by the inclusion of parallel blanks.

Estimation of total nitrite and nitrate concentration

Nitric oxide (NO), a reactive free radical is generally oxidized to NO× (nitrite-NO_2^-/nitrate-NO_3^-). The total nitrite and nitrate concentration was measured using the indirect method of Sastry et al. [44] in liver, kidney, hypothalamus, and testis using acidic Griess reaction for color development after the reduction of nitrate with copper-cadmium alloy and deproteinization. In brief, the reaction mixture was prepared by adding 100 µl of sample or standard (KNO_3), 400 µl of carbonate buffer, and a small amount (0.15 g) of activated copper-cadmium alloy fillings (washed in buffer and dried on a filter paper) in the tube. The reaction mixture was then incubated at room temperature for 1 h with thorough shaking. The reaction was stopped by the addition of 100 µl of 0.35 M sodium hydroxide (NaOH), followed by 400 µl of 120 mM zinc sulphate ($ZnSO_4$) solution under vortex, and allowed to stand for 10 min. The tubes were then centrifuged at 8000 rpm for 10 min. 100 µl aliquots of the clear supernatant were transferred into the wells of a microplate (in quadruplicate) and Griess reagent (50 µl of 1.0% sulphanilamide prepared in 2.5% orthophosphoric acid and 50 µl of 0.1% N-naphthylethylenediamine prepared in distilled water) was added to it with gentle mixing. After 10 min, the absorbance was measured at 545 nm against a blank (containing the same concentrations of ingredients but no biological sample) in a microplate reader (MS5605A, ECIL, Hyderabad, India) using the path check option.

Lipid peroxidation assay

Level of lipid peroxides were measured by estimation of malondialdehyde (MDA), end product of lipid peroxidation using the method of Ohkawa et al. [45] with a slight modification. MDA reacts with thiobarbituric acid (TBA) to yield a colored compound. Briefly, to 100 µl of tissue homogenate, 50 µl of 0.8% butylated hydroxytoluene, 750 µl 0.8% of TBA, 750 µl of 20% acetic acid (pH 3.5), and 100 µl of 8% SDS were added. The reaction mixture was then incubated at 95°C in water bath for 1 h. After this, the mixture was cooled down by keeping on ice for 15 min and then centrifuged at 2000 rpm for 10 min. Absorbance was taken at 532 nm and the results was expressed as nano moles MDA/mg protein.

Antioxidant enzymes assay by native PAGE

ROS-scavenging enzymes: SOD, CAT, and GPx activities were determined by nondenaturing polyacrylamide gel electrophoresis (Native PAGE). SOD assay was performed using 12% native gel slab in Tris-glycine buffer (pH 8.3) at a constant voltage of 100 V. In case of liver 20 µg, kidney 30 µg, hypothalamus 35 µg, testis 30 µg, and for plasma 30 µg of protein was loaded in each lane. After electrophoresis, the gel was subjected to substrate-specific staining of SOD bands as described by Beauchamp and Fridovich [46]. The staining mixture consisted of 2.5 mM NBT, 28 µM riboflavin, and 28 mM TEMED. After 30 min incubation in the dark, gel was exposed to a fluorescent light to develop achromatic bands against dark-blue background corresponding to SOD protein in the gel.

Active level of CAT was determined by the method of Sun et al. [47]. For this, sample containing 15 µg proteins for liver and kidney, 25 µg for hypothalamus and testis, and for plasma 30 µg protein were electrophoresed on 7.5% Native PAGE. To developed specific bands of CAT, gels were soaked for 10 min in 0.003% H_2O_2 and then incubated in a staining mixture consisted of 1% potassium ferricyanide and 1% ferric chloride. Achromatic bands of catalase appeared against a blue–green background.

GPx activity was assayed using the method of Lin et al. [48]. After 10% nondenaturing PAGE containing 30 µg protein in case of liver, 40 µg for kidney, 50 µg for hypothalamus, 40 µg for testis and plasma, the gels were incubated in a staining mixture composed of 50 mM Tris-Cl buffer (pH 7.9), 3 mM GSH, 0.004% H_2O_2, 1.2 mM NBT, and 1.6 mM PMS for 45 min. Achromatic bands corresponding to GPx activity appeared against a violet–blue background.

Enzyme activity staining after native PAGE was repeated at least five times and the intensity of bands in all the cases were quantitated by gel densitometry using Image J vs 1.36 software (NIH, USA).

Biochemical estimation of 3β HSD

3β-hydroxysteroid dehydrogenase (3β HSD) enzyme activity in testis of mice was assayed using the method of Shivanandappa and Venkatesh [49]. In brief, 10% homogenate was prepared in 0.1 M Tris-HCI buffer (pH 7.8). The homogenate was centrifuged at 12,000 g at 4°C and the supernatant was used as the source of enzyme. The reaction mixture contained 0.1 M Tris-HCI buffer (pH 7.8) containing 500 µM NAD, 100 µM DHEA as substrate and 50 µl supernatant in a total volume of 3 ml and incubated at 37°C for 1 h. The reaction was stopped by the addition of 2 ml of phthalate buffer (pH 3) and the absorbance was noted at 490 nm. The enzyme activity was calculated from the standard curve of NADH and expressed as nano moles NADH formed/min/mg protein.

Enzyme immunoassay for testosterone

The level of testosterone in the plasma was measured directly using commercial "DS-EIA-Steroid-Testosterone" kit (DSI S.r.l., Saronno (VA), Via A, Volonterio 36a, 21047, Italy) according to the manufacturer's instructions.

Immunohistochemistry of i-NOS

Immunohistochemistry for i-NOS was carried out on paraformaldehyde-fixed paraffin-embedded testis sections using

polyclonal antibodies. Immunohistochemistry was performed in a two-step procedure using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). In the first step, after initial deparaffinization in xylene and rehydration in graded series of alcohol, slides were incubated with rabbit-i-NOS antisera (dilution 1:250) for 24 h in humid chamber [50]. In second step, slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins for 30 min. Finally, diaminobenzidine hydrochloride was used as a chromogen molecule for the immunological detection.

Immunofluorescence of 3β HSD: Confocal microscopy

Testis slides were also processed for immunofluorescence to check the expression of rate-limiting enzyme 3β HSD using goat-3β HSD antisera (1:500) for 24 h. Immunological reactivity was checked using TRITC-conjugated secondary antisera. To confirm the subcellular localization and alteration in the expression of 3β HSD in the cytoplasm of testicular Leydig cells, we have proceeded for Confocal microscopy for immunofluorescent detection of 3β HSD.

Statistical analysis

Data are presented as the mean ± standard deviation. For statistical analysis, Student's *t*-test was performed using SPSS software to compare between the means of the different parameters of the control and experimental groups. A *p*-value of less than 0.05 was considered significant. Since the study was repeated, statistical analysis was done by pooling the data sets of both the experiments.

Results

Histology

We observed marked alterations in the histoarchitecture of the testis of low-level 2.45-GHz MW-irradiated group of mice as compared to those of control group testis in which all the seminiferous tubules showed normal histological features of "full breeding condition". As compared to that of control, a significant reduction was observed in the seminiferous tubule diameter of the testis of exposed group of mice. Further, in addition to non-uniform degenerative changes in the seminiferous tubules, distorted Leydig cells were also observed in the testis of MW-exposed mice. Affected seminiferous tubules in the testis of MW-irradiated mice showed damaged seminiferous epithelium, detachment of the spermatogonial cells from the tunica propria resulting in the formation of a peripheral gap and depletion of germ cells leads to randomized gap formation within some seminiferous tubules (Figure 2).

Sperm count and sperm viability

A significant decrease in the number of sperms ($p < 0.001$) and their viability ($p < 0.01$) was observed in the cauda epididymis of MW-irradiated group of mice compared to that of control. The numbers of dead sperms were found to be increased significantly in exposed group of mice when checked by eosin-negrosin staining (Figure 3).

ROS level

The overall oxidative status in liver, kidney, hypothalamus, and testis homogenates was assessed by measuring total ROS production with a DCFH-DA probe. We observed a significant elevation in ROS production indicated by increased fluorescence intensity in the liver ($p < 0.001$), kidney ($p < 0.01$), hypothalamus ($p < 0.001$), and testis ($p < 0.001$) of MW-exposed group of mice as compared to that of control (Figure 4).

Total nitrite and nitrate concentration

To check whether low-level 2.45-GHz MW radiation cause nitrosative stress via increasing RNS, we measured NO level in liver, kidney, hypothalamus, and testis. In MW-irradiated group of mice, a significant increase in total nitrite and nitrate concentration was observed in liver ($p < 0.01$), kidney ($p < 0.01$), hypothalamus, and testis ($p < 0.05$), compared to that of the control group (Figure 5). These results indicate that the level of NO has increased in these tissues after MW exposure and thus is responsible for generating nitrosative stress.

Lipid peroxidation

It is well documented that oxidative stress induced by the overproduction of free radicals result in lipid peroxidation, therefore we next examine MDA level, the end product of lipid peroxidation which increases during oxidative stress. In the present study, a significant increase has been observed in MDA level of MW-exposed mice in different tissues compared to that of control (Figure 6). Highly significant increase was found in case of liver ($p < 0.001$) and kidney ($p < 0.001$) than hypothalamus ($p < 0.05$) and testis ($p < 0.01$).

Antioxidant enzymes activity

Next we have checked the alteration in the activity of antioxidant enzymes: SOD, CAT and GPx in different tissues by performing native gel (Figure 7). The nonthermal MW exposure increased free radical load and altered the activity of ROS-scavenging enzymes: SOD, CAT and GPx. These antioxidant enzymes combinatorially play a major role in maintaining the intracellular concentration of ROS by forming the first line of defense against ROS.

SOD activity

SOD activity reduced in all different tissues of 2.45-GHz MW-radiation exposed group of mice when compared to that of control (Figure 7a). The SOD activity showed a highly significant decrease in liver ($p < 0.001$), kidney ($p < 0.01$), hypothalamus ($p < 0.001$), and testis ($p < 0.001$) compared to that of control.

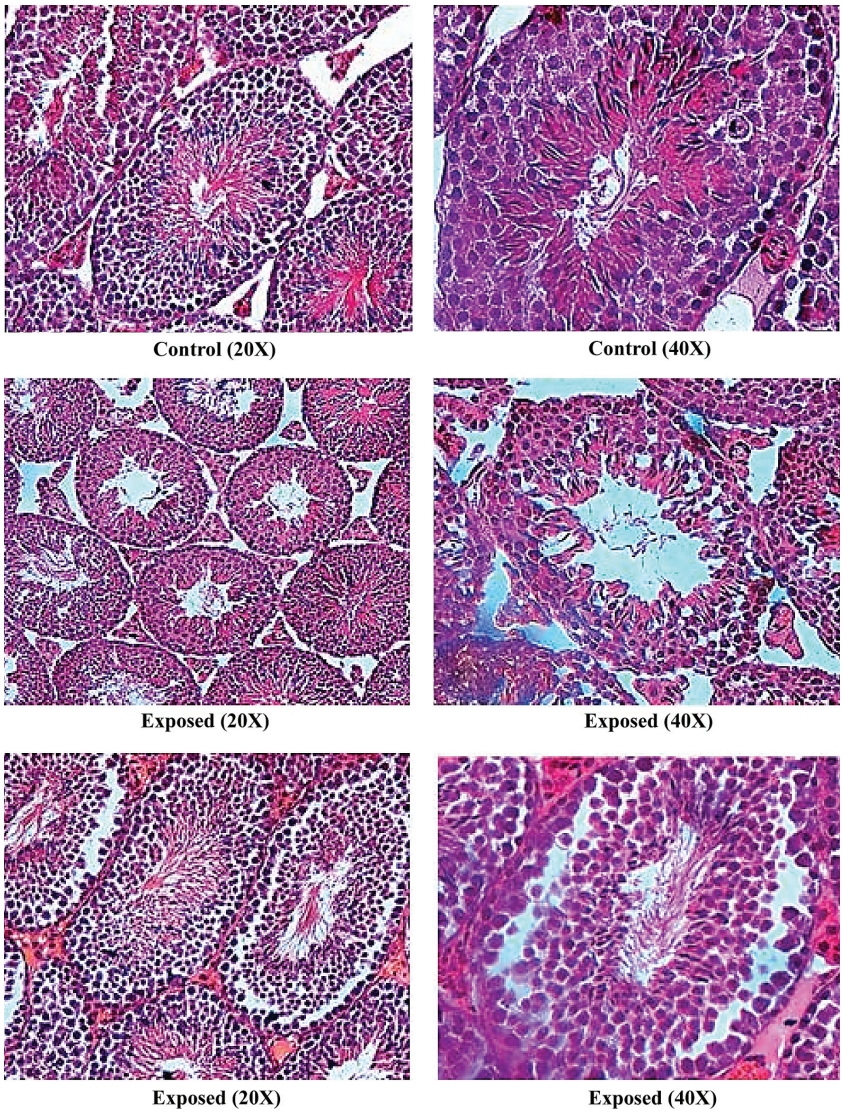

Figure 2. Transverse Section of testis of microwave-irradiated mice (H&E staining). Note significant decrease in the seminiferous tubule diameter and damaged seminiferous epithelium as well as degenerated Leydig cells in the testis of exposed mice (middle and lower panel) compared to that of control (upper panel) Germ cell of exposed mice have a degenerated appearance in the epithelium. Formation of gaps were also seen between the peripheral layers of spermatogonial cells in the seminiferous tubules of nonthermal low-level 2.45-GHz microwave-irradiated mice.

Catalase activity

Similar trend of decrement was also observed in CAT activity (Figure 7b). We found that CAT activity decreased significantly in all different tissues, that is, liver ($p < 0.001$), kidney ($p < 0.01$), hypothalamus ($p < 0.001$), and testis ($p < 0.001$) of MW-irradiated mice when compared to that of the control group.

GPx activity

GPx activity of both the isoforms (GPx1 and GPx2) was found to be decreased significantly ($p < 0.001$) in liver and kidney of MW-exposed mice as compared to that of the control; however, in the hypothalamus and testis GPx 1 and GPx 2, respectively, were found to be decreased significantly. On the other hand, in plasma, only one isoform (GPx1) was evident which did not show any difference between the control and exposed mice (Figure 7c & d).

3β HSD activity

It was found that 2.45-GHz MW radiation caused a statistically significant decrease in the activity of testicular 3β hydroxysteroid dehydrogenase (3β HSD) ($p < 0.01$), a crucial enzyme in the steroid biosynthesis pathway, compared to that of control (Figure 8A).

Plasma testosterone

Both oxidative and nitrosative stress has been shown to potently reduce testosterone production *in vivo*. Therefore, we next measure the plasma testosterone concentration. It has been observed that plasma testosterone level decrease significantly ($p < 0.001$) in the MW-irradiated

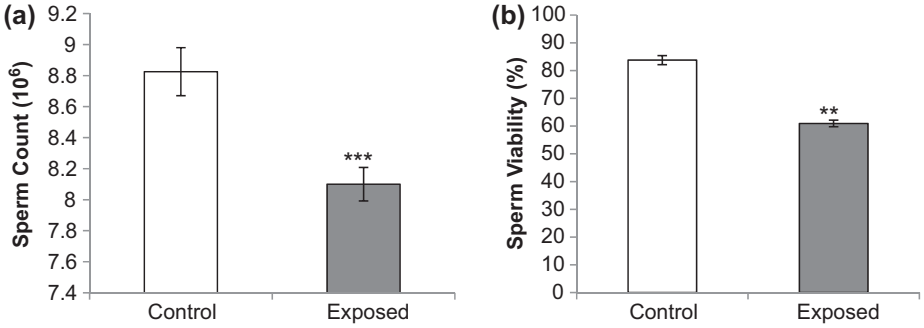

Figure 3. Effect of 2.45-GHz microwave (MW) irradiation on the (a) sperm count and (b) sperm viability of mice. MW irradiation significantly decrease the number of sperm along with increased number of dead sperm compared to that of control. Data are presented as mean ± standard deviations ($n = 15$). Significance of difference from control, $^{**}p < 0.01$, $^{***}p < 0.001$.

group of mice as compared to that of the control group (Figure 8B).

Immunohistochemistry of i-NOS

Nitric oxide (NO), which is a free radical gas as well as a ubiquitous signaling molecule produced by NO synthases, has been proposed to mediate many physiological functions. It has been proposed that the involvement of NO in stress is due to activation of testicular inducible i-NOS. To check whether the tissue-specific synthesis of NO is induced by the MW irradiation, immunohistochemical localization of i-NOS has been performed. Result shows a significant increase in i-NOS-immunoreactivity in the spermatogonial cells of seminiferous tubules and Leydig cells of MW-irradiated mice testis compared to that of control (Figure 9).

Immunofluorescence of 3β HSD

We also observed a significant decrease in the expression of 3β HSD, a key enzyme in steroidogenesis pathway, in the cytoplasm of testicular Leydig cells of exposed group of mice (Figure 10).

Discussion

Present study clearly elucidated that low-level 2.45-GHz MW radiation with power density = 0.029812 mW/cm^2 and SAR = 0.018 W/Kg has negative impact on male reproductive system. These experimental findings indicate that MW irradiation exerts a wide range of adverse effects on testicular functions. It affects the testicular morphology by decreasing the diameter of seminiferous tubules, degeneration of seminiferous epithelium, and formation of peripheral gaps in some tubules by the detachment of spermatogonial layer from the tunica propria. These changes including germ cell degeneration, reduced sperm counts, and viability may occur as the consequence of reduced testosterone concentrations in MW-exposed group of mice, since high level of testosterone is essential for normal spermatogenesis as well as for the maintenance of structural morphology and normal physiology of seminiferous tubules [51,52]. Yan and group also reported that long-term exposure to low-intensity MW radiation evidently increased the sexual dysfunction rate in humans [53]. During spermatogenesis, testosterone is also required for the attachment of

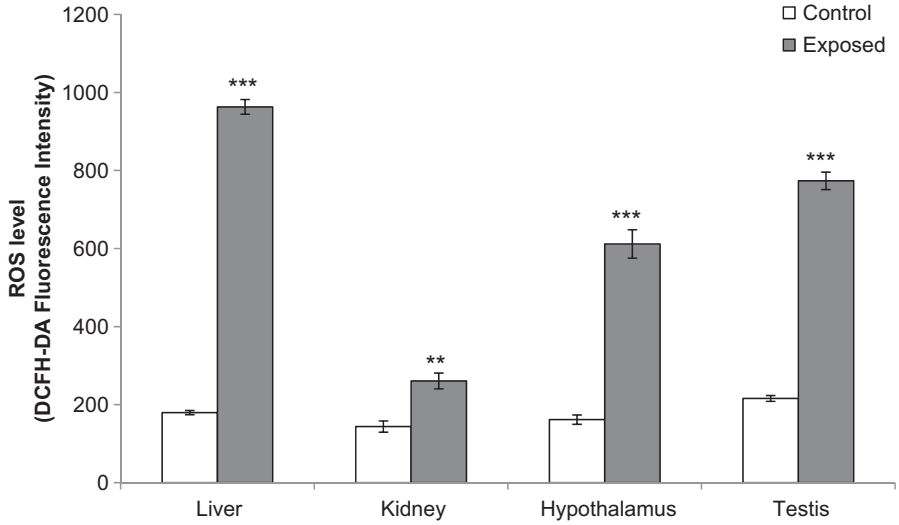

Figure 4. Increased DCFH-DA fluorescence (measure of ROS) was observed in liver, kidney, hypothalamus, and testis of microwave-irradiated mice as compared to that of control. Highly significant increase was observed in all the tissues. Results were expressed as mean ± standard deviations ($n = 15$). Significance of the difference was determined by using the two-tailed Student's t test. $^{**}p < 0.01$ and $^{***}p < 0.001$ significance of difference from control.

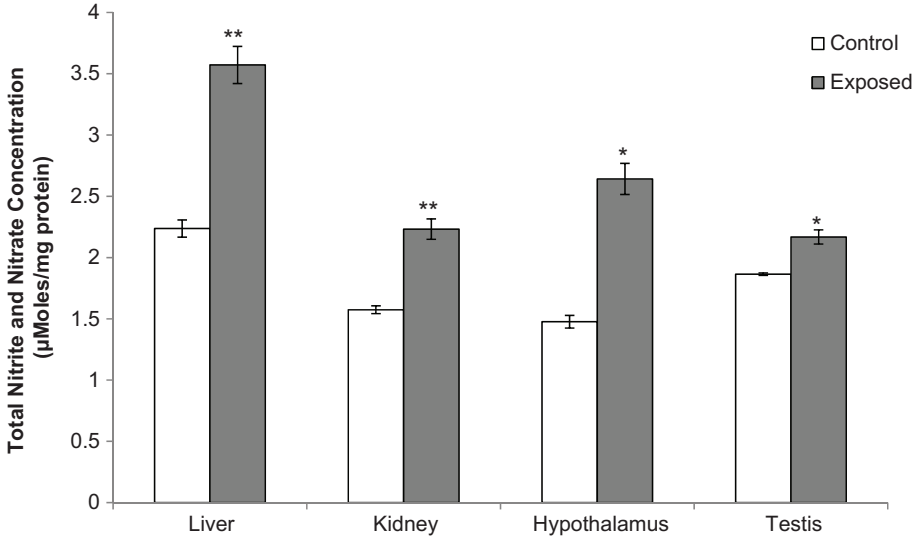

Figure 5. MW-irradiated mice showed a significant increase in total nitrite and nitrate concentration in liver, kidney, hypothalamus, and testis ($n = 15$). Values are expressed as mean ± standard deviations.*$p < 0.05$ and **$p < 0.01$ significance of difference from control.

different generations of germ cells in seminiferous tubules and therefore decreased testosterone concentration may lead to detachment of germ cells from seminiferous epithelium and cell death by initiating germ cell apoptosis [54,55]. Our findings are consistent with previous studies which suggest that MW radiation can negatively affect the quality of sperm which can potentially impair the reproduction in the humans and rodents [56,57]. Although, some researchers have reported that acute MW exposure can have direct effect on the seminiferous tubule epithelium through increase in testicular temperature but, the power density and SAR values used in these studies were too high [18,19,58] and greater than the present study. MW exposure at power density = 0.029812 mW/cm^2 and SAR = 0.018 W/Kg does not cause any elevation in the ambient temperature of the animal cage as well as rectal temperature of mouse and hence eliminates the possibility of thermal effects of MW exposure on spermatogenesis and sperm count and viability.

With a significant elevation in ROS, RNS, and MDA level in liver, kidney, hypothalamus, and testis, we also observed a significant decrease in ROS-scavenging enzymes (SOD, CAT, and GPx) in exposed group of mice. These findings are consistent with our previous study in female mice which demonstrated that mice exposed to 2.45-GHz (CW) MW irradiation is responsible for generating extensive free radical load as evident from increased ROS/RNS and reduction in antioxidant enzyme activities [16]. The kidney is a major potential route for the absorption of hazardous materials encountered in the environment. It helps in the excretion of waste products of metabolism and drugs. It regulates the homeostasis of body water as well as performs endocrine (hormonal) functions. As a multifunctional organ, liver takes part in uptake, metabolism and excretion of nutrients. It helps in the excretion of endogenous wastes and xenobiotics, synthesis and secretion of plasma proteins, clotting factors. It also maintains metabolic homeostasis (glucose and lipids) and assists in the digestion of fat (via bile). The alteration/

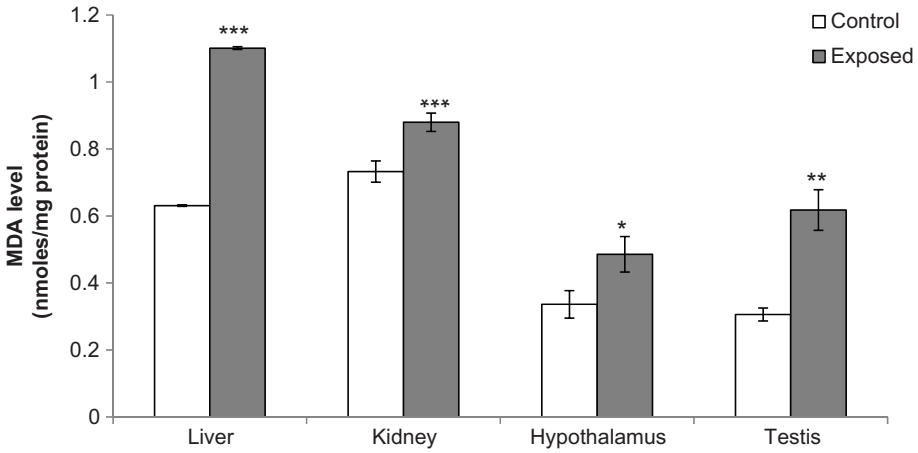

Figure 6. 2.45-GHz MW irradiation result in increased lipid peroxidation. Significant increase in malondialdehyde (MDA) level, end product of lipid peroxidation, was observed in different tissues of MW-exposed mice. Values were expressed as mean ± standard deviations ($n = 15$). Data were analyzed using the two-tailed Student's t-test. Significance of difference from control,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

significant increase in oxidative and nitrosative stress parameters (free-radical load, ROS, NO, and MDA level) and significant decrease in the activities of antioxidant enzymes in kidney and liver suggests the ill effects of MW at the organ as well as the system level. These continuously produced ROS are scavenged by SOD, GPx, and CAT. Under some circumstances, these endogenous antioxidative defenses are likely to be perturbed as a result of overproduction of oxygen radicals, inactivation of detoxification systems, consumption of antioxidants, and failure to adequately replenish antioxidants in tissue. It has been demonstrated in numerous studies that ROS are directly involved in oxidative damage of cellular macromolecules such as lipids, proteins, and nucleic acids in tissues. The exact mechanism of MW-interactions is not yet clearly understood, although few studies have suggested the involvement of lipid peroxidation, free radical formation, and biochemically induced oxidative stress. Since, in addition to its reproductive effects MW radiation also affects liver and kidney, present study suggests that MW exposure may interfere with the overall functions of the organ system.

With reference to the reproductive system, it appears that in testis, over production of free radicals and decreased anti-oxidant status are responsible for causing toxic effects on the testicular physiology. SOD and CAT are enzymatic antioxidants which inactivate the superoxide anion ($O_2^{\bullet -}$) and peroxide (H_2O_2) radicals by converting them into water and oxygen. GPx is the final member of the antioxidant triad which is involved in the reduction of hydrogen peroxides using glutathione as an electron donor. Therefore, as a consequence of MW radiation-induced decrease in ROS-scavenging enzymes: SOD, CAT, and GPx, there is spontaneous increase in toxicity due to elevation of free radicals superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$),

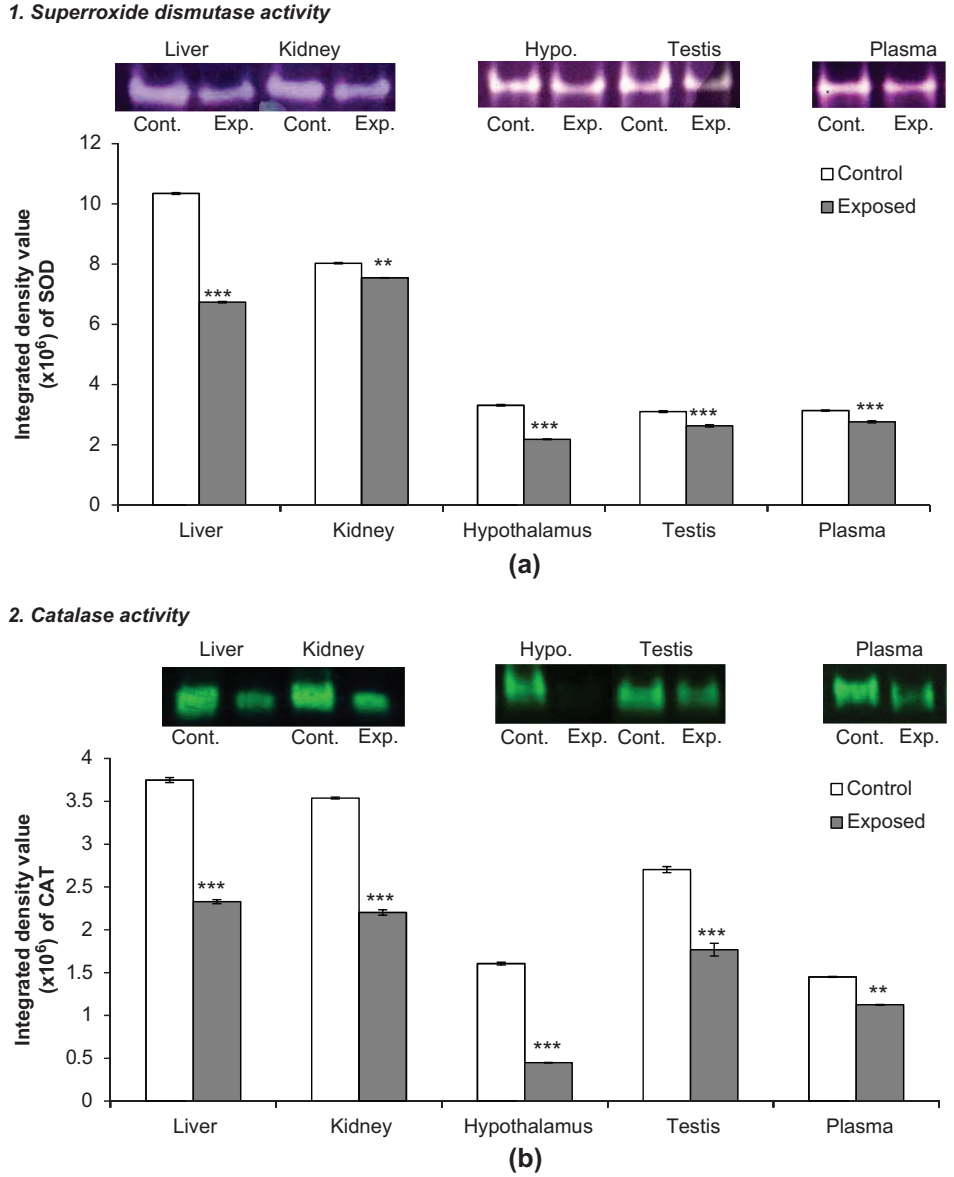

Figure 7. Changes in Antioxidant enzymes activities—SOD (a), CAT (b) and two isoforms of GPx (GPx 1-c and GPx 2-d) of 2.45-GHz microwave-irradiated mice demonstrated by native gel. The intensity of bands in all the cases is quantified by gel densitometry. Values are expressed as mean ± standard deviation. Significance of difference from control $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

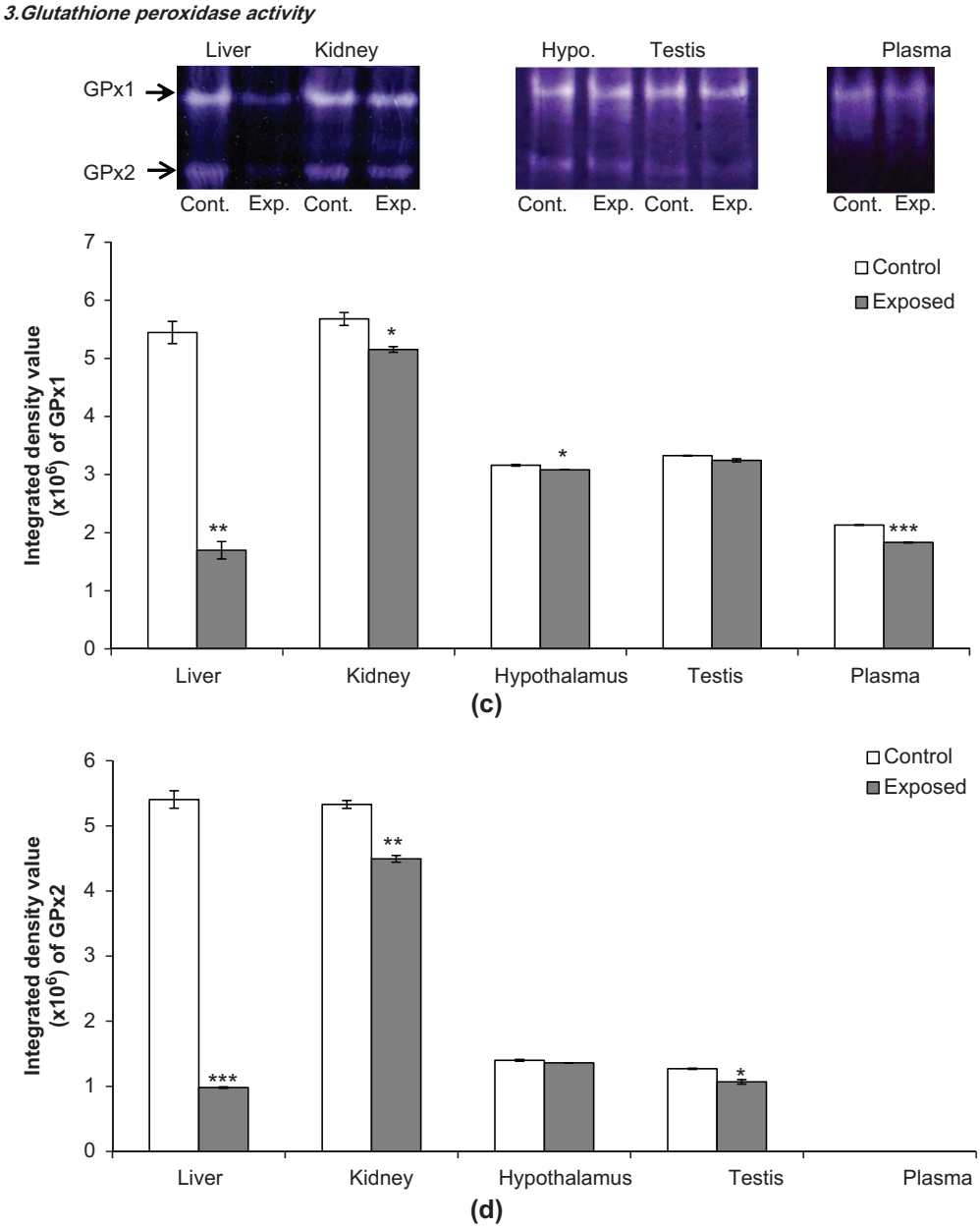

Figure 7. (*Continued*)

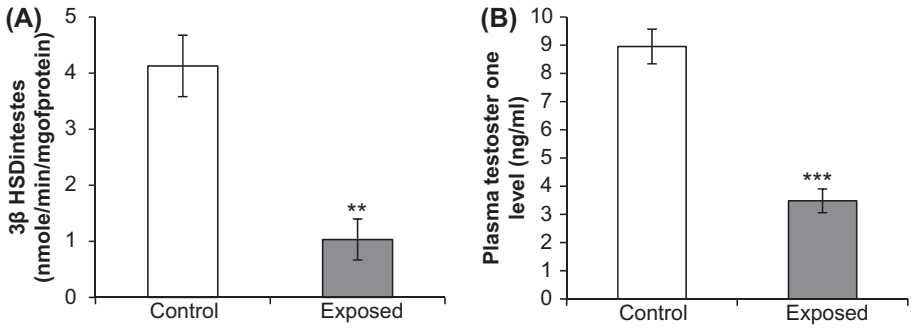

Figure 8. Effect of 2.45-GHz microwave irradiation on (A) testicular 3β HSD and (B) Plasma Testosterone concentration. Highly significant decrease was observed in 3β-HSD activity, a key enzyme in steroidogenesis pathway. Plasma testosterone level is also found to be significantly reduced in MW-exposed group. Values are expressed as mean ± standard deviation. Significance of difference from control, $^{**}p < 0.01$, $^{***}p < 0.001$.

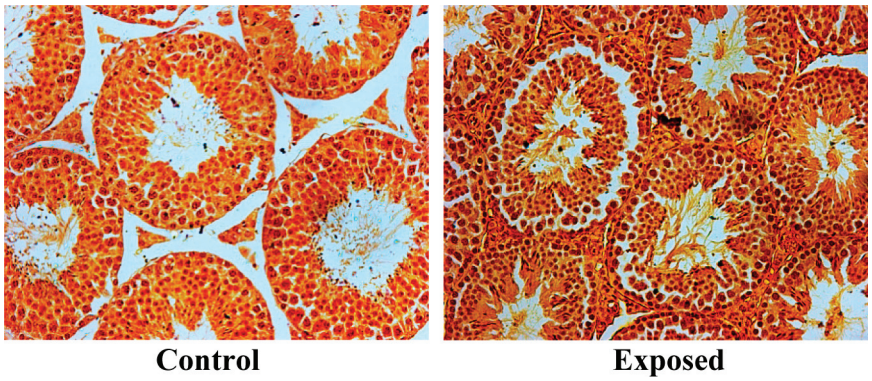

Figure 9. Immunohistochemistry of i-NOS in mice testis. Note significantly increased i-NOS immunostaining in the spermatogonial cells of seminiferous tubules and Leydig cells of microwave (MW)-irradiated mice testis compared to that of control. 2.45-GHz nonthermal MW exposure induce the activation of testicular i-NOS and subsequently raising the production of NO which is a free radical gas as well as main causative agent of nitrosative stress, resulting havoc free radical load.

etc. in the body. This results in increased lipid peroxidation in different tissues. Desai et al. [59] and Agarwal et al. [60] showed a possible mechanism for increased ROS generation in various cellular targets. MW radiation stimulates plasma membrane NADH oxidase. SOD and CAT remove $O_2^{\bullet -}$ generated by NADPH oxidase and play a major role in decreasing lipid peroxidation and protecting spermatozoa against oxidative damage [61]. Evidences show a link between deficient SOD and CAT activity, and male infertility [62–66]. GPx must play an important protective role against oxidative attack since its specific inhibition *in vitro* using mercaptosuccinate leads to a large increase in sperm lipid peroxidation [67]. Male factor infertility linked with a reduction in seminal plasma [68] and spermatozoa GPx activity [69] further supports an important role for this enzyme in male fertility.

Low amount of controlled endogenous ROS generation by spermatozoa play a significant role in inducing sperm capacitation/acrosome reaction and acquisition of sperm-fertilizing ability [28,70]. Thus, although physiological levels of ROS are required for spermatogenesis, an excessive production of ROS resulting from low-level 2.45-GHz MW radiation appears to have deleterious effects on testicular function. High generation of ROS accompanied by

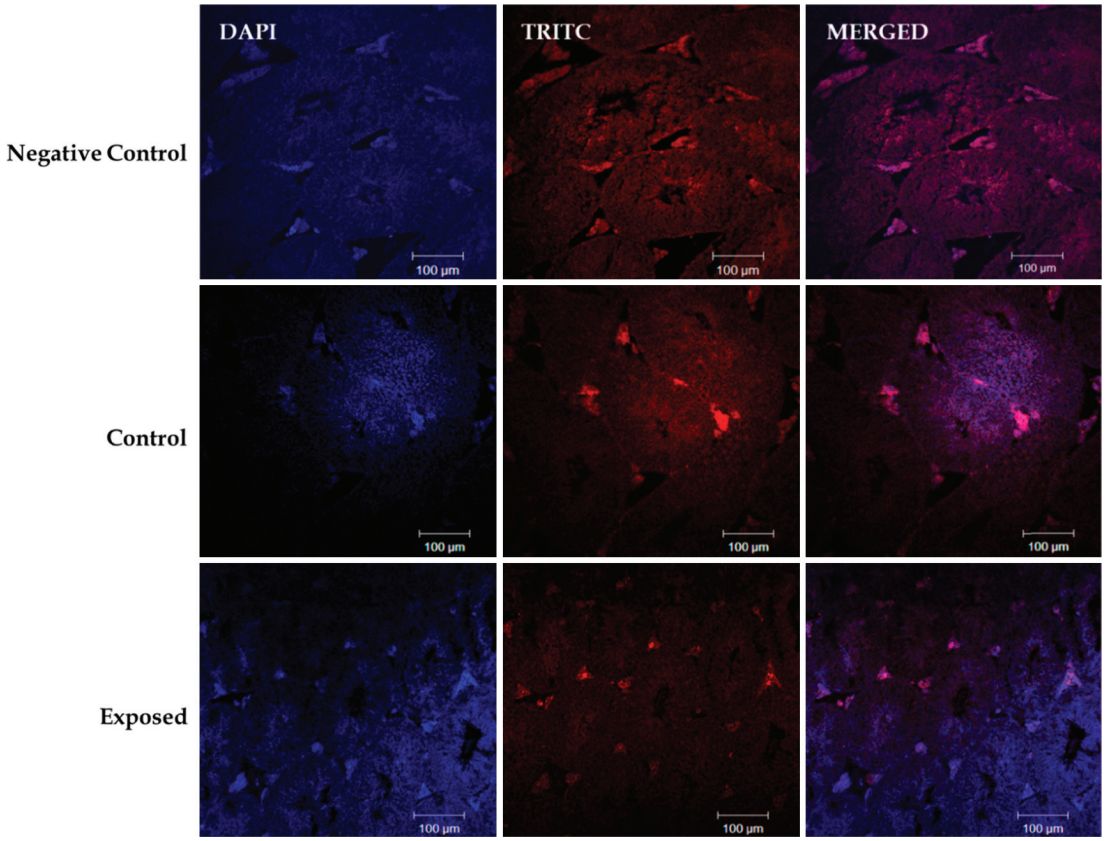

Figure 10. Confocal microscopy showing immunofluorescence of 3β-HSD in testicular Leydig cells of MW-irradiated mice. Note significantly decreased expression of 3β-HSD enzyme, one of the prime enzyme in the steroidogenesis pathway, which play a key regulatory role in testicular androgenesis, in the cytoplasm of testicular Leydig cells of low-level 2.45-GHz MW-exposed mice compared to that of control.

low scavenging antioxidant levels in different tissues will induce a state of oxidative stress. Since testicular membranes are rich in molecules such as fatty acids (i.e., lipids), which are prone to oxidative injury, it is reasonable to consider that lipid peroxidation (i.e., damage to the cell membranes) may contribute to the gonadal dysfunction that occurs as a result of low-level MW irradiation. Since, overproduction of free radicals by MW exposure results in lipid peroxidation, which may cause rupture of the cell as well as nuclear membrane, it is suggested that degeneration of seminiferous epithelium and disarray in cellular organization of seminiferous tubules is the outcome of MW-induced oxidative/nitrosative stress in the testicular environment. Free radicals generated after MW irradiation also react with the polyunsaturated fatty acid-rich spermatozoa resulting in peroxidation which finally leads to sperm destruction causing reduced motility and viability. Studies also suggest that elevated ROS level endanger sperm motility, viability, and function by interacting with membrane lipids, proteins and nuclear and mitochondrial DNA [71–74]. Falzone et al. [75] demonstrated that mitochondrial membrane potential of the human sperm cells was decreased after 900 MHz mobile phone EMR. Further, Erogul and group [12] reported that semen samples from 27 healthy human volunteers exposed to MW radiation emitted from an active GSM cell phone at 10 cm distance caused a statistically significant decrease in forward sperm movement, as well as a reduction in total number of motile sperm. It has been observed that such radiation can reduce the fertilizing potential of men [10,11,13] and may lead to oxidative stress via increasing ROS level and decreasing total antioxidant capacity (TAC) score in human semen [14].

2.45-GHz MW irradiation at SAR = 0.018 W/Kg significantly decreased the steroidogenic enzyme 3β-HSD in the testis as well as plasma testosterone level. We suggest that MW radiation-induced oxidative and nitrosative stress adversely affects steroidogenesis and thus spermatogenesis which may lead to infertility in chronic condition. Since testicular functions and the process of spermatogenesis is hormonally regulated by a negative feedback loop, adverse effects of MW radiation on HPG axis cannot be ruled out. MW radiation-induced elevation in hypothalamic ROS and RNS may disrupt the hormonal communication of the neuroendocrine axis which disturbs the steroidogenesis and spermatogenesis process of testis. Present findings are consistent with other studies which reported that MW exposure disrupts the histological architecture of seminiferous tubules, reduces the Leydig cell population, and testosterone concentration in rats [76]. In addition to deleterious effects of ROS and NO at the hypothalamus level it is also possible that increased NO level in testis following 2.45-GHz MW irradiation may decrease steroidogenesis by affecting Leydig cells directly and thus spermatogenesis in male. NO has been also shown to potently reduce testosterone production both *in vivo* [77] and *in vitro* [78,79]. Thus, similar to ROS, although optimum level of NO has beneficial effects on reproductive performance, its over production may affect steroidogenesis/spermatogenesis adversely [80,81].

Based on adverse testicular morphology and physiology as well as increased ROS and RNS at both the central and peripheral level, we suggest that 2.45-GHz MW irradiation with power density = 0.029812 mW/cm^2 and SAR = 0.018 W/Kg has a negative effect on testicular function (testicular steroidogenesis and spermatogenesis) through the induction of oxidative and nitrosative stress. It is well known that mice are metabolically more active than humans, providing an indication of possible effects on human reproductive system at similar power density and SAR value. Since these findings may be extrapolated to humans, it is high time to have the awareness based on experimental reports regarding the possibility of long-term deleterious effects of MW radiation.

Conclusion

Present experimental findings clearly indicate that MW irradiation impairs male reproductive system via inducing oxidative as well as nitrosative stress. It is obvious that low-level 2.45-GHz MW irradiation produces degenerative changes in the spermatogonial cells and inhibits testosterone production by increasing ROS and RNS production and disturbing testicular antioxidant status. We also suggest that MW irradiation acts as an environmental toxicant that stimulates lipid peroxidation. Further, oxidative and nitrosative stress induced at the central and peripheral level as a consequence of low-level 2.45-GHz MW exposure diminishes the steroidogenic capacity of the testis by decreasing 3β-HSD activity as well as decreased plasma testosterone level, resulting in decreased spermatogenesis. This experimental report also suggests that chronic exposure to nonthermal MW radiation may include deleterious changes in reproductive performance, which may lead to infertility following long-term exposure. Further, similar to IR, MW-induced nonionizing radiation may also impose health risk. Hence, long-term continuous exposure to man-made nonionizing MW radiation even at low level should also be minimized.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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